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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : G01N 33/74, 33/574, 33/50, C12N 5/10, A61K 51/08 — A1

(11) International Publication Number: **WO 98/08100**

(43) International Publication Date: 26 February 1998 (26.02.98)

(21) International Application Number: PCT/CA97/00592

(22) International Filing Date: 20 August 1997 (20.08.97)

(30) Priority Data:
60/024,409 23 August 1996 (23.08.96) US

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(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: AGONIST-DEPENDENT INTERNALIZATION OF HUMAN SOMATOSTATIN RECEPTORS TYPES 1-5

(57) Abstract

The present invention relates to the uses of human somatostatin receptors types 1-5 in the diagnosis and/or treatment of diseases and more particularly to tumor cells, e.g. breast cancer and in cases of Alzheimer's. The present invention relates to (i) recombinant host cells (CHO-K1 cells) individually expressing the 5 hSSTR subtypes; (ii) the use of these cells as tools for testing the ability of synthetic SST agonists or antagonists to induce receptor internalization or upregulation; (iii) the use of the internalization property of hSSTR subtypes to target tumors for selective targeted destruction. For instance, subtypes such as hSSTR3 and 5 which are extensively internalized could be targeted with selective α or β-emitting SST radioligands for radiotherapy of, for example, breast cancers which display a rich concentration of these receptors; (iv) the use of the ability of hSSTR1 (in particular) and hSSTR2 and 4 (to a lesser extent) to be upregulated at the cell surface upon prolonged treatment with agonist as a test for increasing the sensitivity of receptor scans for detection and diagnosis of tumors and inflammatory conditions; (v) the use of the differential ability of the hSSTR subtypes to be upregulated or internalized as a means for producing enhanced images in receptor scans by subtraction analysis to recruit, for example, SSTR1 selective to the membrane whilst internalizing others such as hSSTR3, hSSTR4, and hSSTR5.

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AGONIST-DEPENDENT INTERNALIZATION OF HUMAN SOMATOSTATIN RECEPTORS TYPES 1-5

BACKGROUND OF THE INVENTION

(a) Field of the Invention

The invention relates to the uses of human somatostatin receptors types 1-5 in the diagnosis and/or treatment of diseases and more particularly to tumor cells and in cases of Alzheimer's.

(b) Description of Prior Art

Somatostatin (SST) occurs naturally as 2 bioactive peptides, SST-14 and SST-28, which exert potent effects on multiple targets including the brain, gut, pituitary, endocrine and exocrine pancreas, adrenal, thyroid, kidneys, and immune cells (Patel YC, 1992, *In: The role of somatostatin: basic and clinical aspects of neuroscience series*, Muller EE et al. (eds), Vol. 4, pp 1-16, Springer-Verlag, Berlin). The cellular actions of SST include the inhibition of hormone and exocrine secretion, as well as modulation of neurotransmission and cell proliferation, and are mediated by a family of G protein coupled receptors (GPCR) termed SSTR1-5 (Patel YC et al., 1995, *Life Sci.*, **57**:1249-1265; Reisine T et al., 1995, *Endocrinology*, **16**:427-442). SSTR1-4 display weak selectivity for SST-14 binding whereas SSTR5 is SST-28 selective (Patel YC et al., 1995, *Life Sci.*, **57**:1249-1265). Long acting SST analogs such as the octa-peptide SMS201-995 (SMS, octreotide) or the hexapeptide MK678 used clinically for diagnosis and treatment of neuroendocrine tumors bind to 3 of the SSTR subtypes 2,3, and 5 (Patel YC et al., 1995, *Life Sci.*, **57**:1249-1265; Reisine T et al., 1995, *Endocrinology*, **16**:427-442). Although the acute administration of SST-14 or SMS produces a diverse range of biological effects, the initial effects diminish with continued exposure to the peptides due to the development

of tolerance (Lamberts SWJ et al., 1996, *N. Engl. J. Med.*, **334**:246-254). Such agonist-dependent desensitization of SSTR responses was initially suggested by studies showing that multiple injections of SST induced tachyphylaxis of islet insulin suppression in the rat (Patel YC et al., 1995, *Life Sci.*, **57**:1249-1265). Subsequent studies revealed that short term exposure of rat anterior pituitary cells or AtT-20 cells to SST led to SSTR desensitization (Patel YC et al., 1995, *Life Sci.*, **57**:1249-1265; Reisine T et al., 1995, *Endocrinology*, **16**:427-442; Reisine T et al., 1983, *Endocrinology*, **113**:811-813).

More prolonged agonist exposure for 24-48 h was shown to upregulate SSTRs in GH4C1 and RINm5f cells (Presky DH et al., 1988, *J. Biol. Chem.*, **263**:714-721; Sullivan SJ et al., 1986, *J. Biol. Chem.*, 261:3571-3577). Agonist-specific desensitization is common to many GPCRs and is associated with receptor phosphorylation, uncoupling of the receptor from G proteins, receptor internalization, and receptor degradation (Dohlman HG et al., 1991, *J. Biol. Chem.*, **268**:337-341). The under-lying mechanisms for SSTR desensitization or upregulation are poorly understood. Agonist-induced uncoupling of SSTRs from G proteins has been shown in AtT-20 cells (Reisine T et al., 1995, *Endocrinology*, **16**:427-442; Reisine T et al., 1983, *Endocrinology*, **113**:811-813). Agonist-dependent internalization of SSTRs has been demonstrated in rat anterior pituitary cells, in rat islet cells, and AtT-20 cells (Morel G et al. 1985, *Endocrinology*, **116**:1015-1020; Amherdt M et al., 1989, *J. Clin. Invest.*, **84**:412-417; Hofland LJ et al., 1995, *Endocrinology*, **136**:3698-3706).

Since normal pituitary and islet cells or their tumor cell derivatives are now known to express multiple SSTR subtypes, it is conceivable that the reported

differences in the downregulation or upregulation of endogenous SSTRs are due to differential regulation of the different SSTR subtypes expressed by these cells (Patel YC et al., 1995, *Life Sci.*, **57**:1249-1265; Reisine T et al., 1995, *Endocrinology*, **16**:427-442).

It would be highly desirable to be provided with the characterization of agonist-induced receptor internalization or upregulation of the 5 human (h) SSTR subtypes that have been individually expressed in stable host cells, such as CHO-K1, and their respective uses thereof for the diagnostic and/or treatment of tumor cells.

SUMMARY OF THE INVENTION

There are 5 somatostatin receptors in humans termed hSSTR1-5 that have now been identified by molecular cloning. SST agonists which bind and activate these receptors and which are currently available for diagnosis or treatment, consist of the natural ligand SST-14 which binds to all 5 hSSTRs, and octapeptide analogs such as Octreotide which bind to the subtypes 2, 3 and 5. By studying the regulation of the 5 hSSTRs separately expressed in CHO-K1 cells, we have found that upon treatment with agonist, hSSTR2, 3, 4 and 5 are rapidly internalized over 60 min. Maximum internalization occurs with hSSTR3 (78%) followed by hSSTR5 (60%). hSSTR4 and hSSTR2 are also internalized but to a lesser extent (29% and 20% respectively). In contrast, hSSTR1 is not internalized. However, when all 5 hSSTRs are exposed to agonist for a long period of time (22 h) as might happen during chronic treatment of patients with SST agonists, we find that hSSTR1 is upregulated at the cell surface by 110%. hSSTR2 and 4 are weakly upregulated by 26% and 22% respectively

whereas the levels of hSSTR3 and 5 do not change at the membrane.

One aim of the present invention is to provide the characterization of agonist-induced receptor internalization or upregulation of the 5 human (h) SSTR subtypes individually expressed in stable CHO-K1 cells and their respective uses thereof for the diagnosis and/or treatment of tumor cells.

Another aim of the present invention is to provide the use of the internalization property of hSSTR subtypes to target tumors for selective targeted destruction. For instance, subtypes such as hSSTR3 and 5 which are extensively internalized could be targeted with a cytotoxic agent in addition to selective α or β emitting SST radioligands for radiotherapy of, for example, breast cancers which display a rich concentration of these receptors.

Another aim of the present invention is to exploit the ability of hSSTR1 (in particular) and hSSTRs 2 and 4 (to a lesser extent) to be upregulated at the cell surface upon prolonged treatment with agonist, as a mechanism for increasing the sensitivity of receptor scans for detection and diagnosis of tumors and inflammatory conditions. For instance, treatment of patients suspected of having a tumor which is SSTR1 positive (e.g. breast or prostate cancer) for 24 h with an agonist for hSSTR1 (e.g. SST-14) should increase the number of surface receptors on the tumors for visualization by subsequent receptor scan with a labeled hSSTR1 agonist. Although somewhat less effective, the same principle could also be used for recruiting hSSTR2 receptors to the cell surface (e.g. by 24 h treatment with SST-14 or Octreotide) prior to their detection by a labeled Octreotide receptor scan.

Another aim of the present invention is to use the differential ability of the hSSTR subtypes to be upregulated or downregulated as a means for producing enhanced images in receptor scans by subtraction analysis to recruit e.g. SSTR1 selectively to the membrane whilst internalizing others e.g. SSTRs 3, 4, 5. For instance, a pituitary tumor expressing predominantly the hSSTR1 subtype surrounded by normal pituitary tissue expressing several hSSTR subtypes (e.g. types 3, 4 5) could be visualized more sharply by receptor scan by treating the patient for 24 h with a hSSTR1 selective analog to enhance SSTR1 receptors and then administering for 1 h agonists that will bind and internalize hSSTR3, 4 and 5 in the surrounding normal tissue prior to scanning with a labeled hSSTR1 agonist.

Another aim of the present invention is to provide for the long-term administration of somatostatin analogs that are selectively targeted on receptors that are expected to be upregulated due to reduced production of somatostatin as a result of disease. For instance, a well known biochemical marker of Alzheimer's Disease is a profound reduction in the production of somatostatin by neurons in the deeper layers of the cerebral cortex. Since occupancy of hSSTRs 3, 4 and 5 leads to their immediate internalization, a deficiency of endogenous SST ligand would produce a state of chronic upregulation of these hSSTR subtypes. Since it is very likely that a disturbance of somatostatin production and the associated changes in its receptors plays a major role in producing symptoms (e.g. cognitive impairment) in Alzheimer's Disease, replacement therapy with selective somatostatin analogs could be targeted towards subtypes such as 3, 4 and 5 in order to normalize their function.

In accordance with the present invention, there is provided recombinant host cells individually expressing the hSSTR1-5 receptor subtypes. These recombinant host cells may be used in a method for quantifying the amount of hSSTR1-5 receptors present on the cells, which comprises the steps of:

a) incubating membrane fractions from host cells expressing one of the five hSSTRs with saturating concentrations of a cytotoxic agent in addition to a radioligand; and

b) determining the amount of hSSTR1-5 receptors present on the cell fractions of step a) in a saturation analysis.

More particularly this method is carried out as follows:

incubating membrane fractions from host cells (e.g. CHO-K1 cells) expressing one of the five hSSTRs with saturating concentrations of a cytotoxic agent (e.g. methotrexate or doxorubicin) in addition to a radioligand (e.g. [^{125}I] Leu^{8}, D-Trp^{22}, Tyr^{25} SST-28) in a saturation analysis.

These recombinant host cells may be used in a method for determining their potency for binding to SST agonists and antagonists by competition analysis by displacement of membrane bound radioligand (e.g. [^{125}I] LTT SST-28) with known amounts of SST-14, SST-28 or other SST agonists.

These recombinant host cells may be used in a method for determining the ability of the expressed hSSTR1-5 to be internalized which comprises the steps of:

a) culturing cells individually expressing SSTR1-5 to about 90% confluency;

b) washing the cultured cells and incubating overnight at 4°C with a binding buffer containing [^{125}I] LTT SST-28;

c) washing the cells of step b) with binding buffer and warming to 37°C to initiate internalization;

d) removing surface-bound radioligand with acid wash; and

e) the internalized radioligand is measured as acid resistant counts in NaOH extracts of acid washed cells and the radioactive fractions are counted in a γ-spectrometer.

More particularly this method is carried out as follows: culturing CHO-K1 cells individually expressing SSTR1-5 in 6 well plates (~1.5x10^6 cells/well) to ~90% confluency. Cells are then washed two times with PBS and incubated overnight at 4°C in 1 X binding buffer (50 mM Hepes, pH 7.5, 2mM $CaCl_2$, 5 mM $MgCl_2$, 5% Ficoll 0.5% BSA, 0.02% PMSF, and 0.02% Bacitracin) with [^{125}I] LTT SST-28 (200,000 cpm) with or without 100 nm SST-14, SST-28, or other SST ligands. Cells are then washed 3 times with binding buffer and warmed to 37°C for different times (0, 15, 30, 60 min.) to initiate internalization. At the end of each incubation, surface-bound radioligand is removed with 1 ml acid wash (20 mM Na acetate pH 5.0) for 10 min. Internalized radioligand can be measured as acid resistant counts in 0.1 N NaOH extracts of acid washed cells and the radioactive fractions are counted in a LKB gamma counter.

These recombinant host cells may be used in a method for determining their ability to be upregulated in response to chronic agonist exposure, which comprises the steps of:

a) determining upregulation of hSSTRs of cells expressing hSSTR1-5 by culturing in a medium with SST agonist;

b) the cells are subjected to acid wash for to remove surface-bound SST; and

c) whole cell binding assay is carried out with a SST radioligand to determine total and nonspecific binding.

More particularly this method is carried out as follows: determining upregulation of SSTRs, CHO-K1 cells expressing hSSTR1-5 by culturing in F10 medium without fetal calf serum with 10^{-7} M SST-14, SST-28, or other SST agonists for 1, 13, 16, 19 and 22 h. Media are then removed and the cells subjected to acid wash for 15 min. at 37°C to remove surface-bound SST. Cells are then washed with 1 X binding buffer and whole cell binding assays are carried out with [^{125}I] LTT SST-28 radioligand for 30 min. at 25°C with or without 10^{-7} M SST-14, SST-28 (to determine total and nonspecific binding).

In accordance with the present invention there is also provided a method of targeted treatment of tumors based on the use of the internalization property of hSSTR3 and hSSTR5 subtypes, which comprises the use of α- or β-emitting SST radioligands for radiotherapy of tumors expressing a rich concentration of the hSSTR3 and hSSTR5 subtypes. The tumors targeted for the treatment are breast cancer tumors or other tumors expressing hSSTR3 and hSSTR5.

In accordance with the present invention there is also provided a method for increasing the sensitivity of receptors scans for the detection and diagnosis of tumors and inflammatory conditions in a patient based on the use of the upregulation property of hSSTR1, hSSTR2 and hSSTR4 subtypes, which comprises the steps of:

a) prolonged treatment of a patient with an agonist of hSSTR1 to increase the number of hSSTR1,

hSSTR2 and hSSTR4 subtypes on the tumor and inflammatory conditions of the patient; and

b) visualization of the tumors and inflammatory conditions of step a) by administering a labeled hSSTR1 agonist.

The agonist used is preferably SST-14.

In accordance with the present invention there is also provided a method for producing enhanced images in receptors scans by subtraction analysis for the detection and diagnosis of a tumor essentially expressing hSSTR1 in a patient based on the use of the upregulation property of hSSTR1 and the internalization property of hSSTR3, hSSTR4 and hSSTR5 subtypes, which comprises the steps of:

a) prolonged treatment of a patient with a hSSTR1 agonist to increase the number of hSSTR1 on the tumor of the patient;

b) administering agonist of hSSTR3, hSSTR4 and hSSTR5 subtypes for the internalization of hSSTR3, hSSTR4 and hSSTR5 subtypes expressed in tissues surrounding the tumor; and

b) visualization of the tumor by administering a labeled hSSTR1 agonist.

The tumor may be of pituitary origin.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 illustrates the time course of internalization of ^{125}I-LTT SST-28 by CHO-K1 cells expressing hSSTR1-5.;

Fig. 2 illustrates the effect of chronic SST treatment of CHO-K1 cells expressing hSSTR1-5 on membrane SSTRs;

Fig. 3 illustrates the confocal immunohistochemical localization of hSSTR1 by rhodamine fluores-

cence of stable hSSTR1 CHO-K1 cells treated with SST-14 (10^{-7} M) for different times; and

Fig. 4 illustrates the comparison of the internalization (short term agonist exposure) and upregulation (chronic agonist exposure) profiles of hSSTR1-5 from Figs. 1 and 2.

DETAILED DESCRIPTION OF THE INVENTION

Agonist regulation of somatostatin receptors (SSTRs) was investigated in stable CHO-K1 cells individually expressing the 5 human (h) SSTR subtypes. hSSTR 2, 3, 4, and 5 displayed rapid agonist-dependent internalization of [^{125}I] LTT SST-28 ligand in a time- and temperature-dependent manner over 60 min. Maximum internalization of radioligand occurred with hSSTR3 (78%) followed by hSSTR5 (66%), hSSTR4 (29%) and hSSTR2 (20%). In contrast, hSSTR1 displayed virtually no internalization. Prolonged agonist treatment led to differential upregulation of some of the SSTRs. After 22 h, hSSTR1 was upregulated at the membrane by 110%, hSSTR2 and hSSTR4 by 26% and 22% respectively, whereas hSSTR3 and hSSTR5 showed little change. Agonist-induced recruitment of hSSTR1 to the membrane was confirmed by immunocytochemistry with hSSTR1 antibodies. These results show that SST regulates all 5 hSSTRs by differential subtype selective internalization or upregulation. Subtype selectivity for internalization and upregulation is inversely related.

MATERIALS AND METHODS

Peptides were obtained as follows: SST-14 (Ayerst Laboratories, Montreal); SST-28 and Leu^8 D-Trp^{22} Tyr^{25} SST-28 (LTT SST-28) (Bachem, Marina Del Ray, CA). Stable CHO-K1 transfectants expressing full length genomic sequences of hSSTR1, 3, 4, and 5 or hSSTR2A cDNA each in the expression vector pRc/CMV

(Invitrogen) were prepared and characterized as previously reported (Patel YC et al., 1994, *Biochem. Biophys. Res. Commun.*, **198**:605-612). Neomycin resistant clones were selected and maintained in Ham's F12 medium containing 10% fetal calf serum and 400 ug/ml G418.

Internalization Experiments

CHO-K1 cells individually expressing hSSTR1-5 were cultured in 6 well plates and studied at ~90% confluency (~1.5 x 10^6 cells/well). On the day of study, medium was removed, the cells washed 2 times with PBS and incubated overnight at 4°C in 1 X binding buffer (50 mM Hepes, pH 7.5, 2 mM $CaCl_2$, 5 mM $MgCl_2$, 5% Ficoll 0.5% BSA, 0.02% PMSF, and 0.02% Bacitracin) with [^{125}I] LTT SST-28 (200,000 cpm) with or without 100 nM SST-14 (for hSSTR1-4) or SST-28 (for hSSTR5). Cells were then washed 3 times with binding buffer and warmed to 37°C for different times (0, 15, 30, 60 min.) to initiate internalization. At the end of each incubation, surface-bound radioligand was removed with 1 ml acid wash (20 mM Na acetate pH 5.0) for 10 min. (Presky DH et al., 1988, *J. Biol. Chem.*, **263**:714-721). Internalized radioligand was measured as acid resistant counts in 0.1 N NaOH extracts of acid washed cells (Presky DH et al., 1988, *J. Biol. Chem.*, **263**:714-721). Radioactive fractions were counted in a LKB gamma counter. Each experiment was repeated 3 times in triplicate.

Chronic Agonist Exposure

CHO-K1 cells expressing hSSTR1-5 were cultured in F10 medium without fetal calf serum with 10^{-7} M SST-14 (hSSTR1-4) or SST-28 (hSSTR5) for 1, 13, 16, 19, and 22 h. Control cells were cultured without SST peptides. At the end of treatment, media were removed and the cells subjected to acid wash for 15 min. at 37°C to

remove surface-bound SST. Cells were then washed with 1 X binding buffer and whole cell binding assays were carried out with ^{125}I LTT SST-28 radioligand for 30 min at 25°C with or without 10^{-7} M SST-14 or SST-28 (to determine total and nonspecific binding). Residual surface binding was calculated as the difference in specific binding between control and experimental groups. Each experiment was repeated 3 times in triplicate and the data were analyzed and plotted using the Inplot Program™ (Graph Pad).

Immunocytochemistry

The effect of chronic SST-14 treatment on membrane hSSTR1 expression was studied by immunocytochemistry. Following treatment of hSSTR1 stable cells with SST-14 for 1, 13, 16, 19, and 22 h, cells were washed 2 times in PBS and fixed for 30 min. at 4°C in 4% paraformaldehyde. After washing with 50 mM Tris-HCl, 1.5% NaCl (TBS) pH 7.4, cells were incubated in 4% normal goat serum in TBS for 1 h followed by incubation with SSTR1 antibody overnight at 4°C. A rabbit polyclonal antibody against the amino terminal peptide sequence ^{49}GTLSEGQ^{55} in hSSTR1 was produced and used at a dilution of 1:100. Preimmune serum and antigen absorbed anti-body were used as controls. Cells were then rinsed 3 times in TBS and incubated for 1 h with rhodamine conjugated secondary antibody at 20°C. After 3 additional washes, cells were mounted with immunofluor and viewed under a confocal microscope.

RESULTS

Fig. 1 shows a comparison of the internalization profiles of radioligand bound to the 5 hSSTR subtypes. In Fig. 1 the maximum percent internalization by each hSSTR is shown in brackets (representative of 3 complete experiments). Four of the subtypes 2, 3, 4 and 5

displayed agonist-dependent internalization of radioligand in a time- and temperature-dependent manner. Internalization of these 4 subtypes occurred at markedly different rates. Maximum internalization of radioligand (78%) occurred in the case of hSSTR3, followed by hSSTR5 (66%) and hSSTR4 (29%). hSSTR2 was weakly internalized to 20% at 60 min whereas hSSTR1 displayed minimal (4%) internalization. Fig. 2 depicts the effect of chronic agonist exposure on surface bound radioactivity. In Fig. 2, the hSSTRs display differential subtype selective upregulation. Numbers in brackets indicate maximum percent increase in membrane binding (representative of 3 complete experiments). Agonist treatment for 22 h led to an upregulation of some of the SSTR subtypes. As in the case of internalization, this process was also subtype-selective. After 22 h, SSTR1 was upregulated by 110%, SSTR2 and SSTR4 were also weakly upregulated by 26% and 22% respectively whereas SSTR3 and SSTR5 showed little change. Fig. 3 illustrates specific SSTR1 immunofluorescence in cells after 0, 13 h, 19 h and 22 h of agonist treatment. In Fig. 3, A - control; B - 13 h; C - 19 h; D - 22 h. Control cells exhibit weak expression of hSSTR1 immunofluorescence at the cell surface (arrow). There is marked increase in labeling at 13 h and 22 h. Specificity of the fluorescence images was determined with preimmune serum, antigen absorbed antibody, and nontransfected CHO-K1 cells. Most cells displayed weak labeling at 0 and 1 h. At 13 h there was noticeably greater expression of hSSTR1 immunoreactivity in the majority of cells. The immunofluorescent labeling increased further at 22 h exhibiting intense labeling of most cells. These results confirm by immunocytochemistry the results obtained by radioligand binding and show that SST is indeed capable of inducing SSTR1.

A comparison of receptor internalization by short term agonist exposure for the 5 SSTR subtypes with receptor upregulation during chronic agonist treatment revealed a reciprocal relationship (Fig. 4). The bars represent maximum percent internalization or upregulation (mean + SE, n = 3). hSSTR3 and 5 which were readily internalized showed no upregulation, whereas hSSTR1 which failed to internalize, displayed marked upregulation. hSSTR2 and 4 exhibited both a moderate level of internalization as well as some upregulation.

DISCUSSION

These results clearly show that SST dynamically regulates all 5 of its receptors at the membrane. hSSTRs undergo agonist-dependent internalization in a time-, temperature-, and subtype-selective manner with the following rank order hSSTR3 > 5, > 4, > 2, > 1. hSSTRs are also differentially upregulated by chronic agonist exposure in a subtype-selective manner. Subtype selectivity for internalization and upregulation is inversely related. Previous studies have suggested that endogenous SSTRs are either recruited to the plasma membrane by agonist as in GH4C1 cells or downregulated through uncoupling and internalization as in AtT-20 cells (Reisine T et al., 1995, *Endocrinology*, **16**:427-442; Reisine T et al., 1983, *Endocrinology*, **113**:811-813; Presky DH et al., 1988, *J. Biol. Chem.*, **263**:714-721). The present findings help to explain these seemingly contradictory observations since upregulation of cell surface binding in GH4C1 cells probably reflects the predominant SSTR1 subtype expressed by these cells and the rapid internalization of radioligand observed in AtT-20 cells is presumably mediated by subtypes such as SSTR5 and 2 which predominate in this cell line (Patel YC et al., 1994, *J. Biol. Chem.*, **269**:1506-1509; Gu Y-Z et al., 1995, *Mol. Pharmacol.*,

48:1004-1014). G protein coupled receptors are internalized both via the classical endocytic pathway involving clathrin-coated vesicles as well as through several other mechanisms such as nonclathrin-coated vesicles (Roettger BF et al., 1995, *J. Cell Biol.*, 128:1029-1041). The clathrin-dependent pathway may be preferentially used for targeting receptors to lysosomes for degradation whereas the nonclathrin vesicles may be more involved in resensitization by recycling the receptor to the membrane following dephosphorylation. All 5 hSSTRs feature the sequence NPXXY at the junction of the 7th TMD and cytoplasmic tail similar to the NPXY internalization motif that has been implicated in mediating the internalization of a number of GPCRs through clathrin-coated pits (Trowbridge IS et al., 1993, *Annu. Rev. Cell Biol.*, 9:129-161). Likewise, the 5 hSSTRs feature a number of phosphorylation sites on serine and threonine residues in the C-tail and cytoplasmic loops that are believed to play a role in receptor sequestration (Patel YC et al., 1995, *Life Sci.*, 57:1249-1265). Thus there are no obvious structural differences that can explain the differential ability or resistance of the 5 hSSTRs to undergo internalization; detailed mutagenesis studies will be required to identify the underlying molecular signals. There are only a few hormones like GnRH, angiotensin II, and prolactin which like SST induce their own receptors (Trowbridge IS et al., 1993, *Annu. Rev. Cell Biol.*, 9:129-161). However, the molecular mechanism underlying homologous receptor upregulation remains obscure. Upregulation of GnRH receptors occurs after 6 or more hours of agonist exposure by a Ca^{2+}-dependent, cycloheximide sensitive process. Our findings that SSTRs are upregulated in transfected cells which lack an endogenous SSTR promoter indicate that upregulation

is signaled directly by the receptors upon ligand binding as also suggested by the finding that upregulation of SSTRs in GH4C1 cells occurs independently of new protein synthesis (Presky DH et al., 1988, *J. Biol. Chem.*, **263**:714-721).

There are functional consequences of the ability of SST to regulate its own receptors. Whilst receptor internalization and subsequent recycling to the membrane form part of the normal desensitization/resensitization response observed in many GPCR systems stimulated short term by hormones or neurotransmitters, the upregulation of SSTRs that occurs after hours of agonist exposure must be largely a pharmacological event. Patients with SST-producing tumors display sustained hypersomatostatinemia which, however, causes minimal symptomatology notably mild steatorrhea, diabetes mellitus, and cholelithiasis secondary to inhibition of pancreatic exocrine secretion, insulin release, and gallbladder contraction (Krejs GJ et al., 1979, *New Engl. J. Med.*, **301**:285-292). Long-term therapy with SMS is also accompanied by signs and symptoms related to these features (Lamberts SWJ et al., 1996, *N. Engl. J. Med.*, **334**:246-254). Based on the present findings, hSSTR1 would be expected to be upregulated in the somatostatinoma syndrome but not by SMS which does not interact with this subtype. hSSTR1 signals through G proteins as well as via G protein-independent pathways and further studies will be necessary to determine the functional state of this receptor and of the other subtypes that are recruited to the membrane by agonist exposure. Although many of the normal responses to SMS are desensitized, it is interesting that hormone producing tumors such as GH adenomas, carcinoid, and VIPomas continue to respond to SMS injections with persistent suppression of hormone

secretion, frequently for several years (Lamberts SWJ et al., 1996, *N. Engl. J. Med.*, **334**:246-254). This suggests a differential regulation of SSTRs in normal tissues and in tumors. Tumors express a high density of SSTRs compared to surrounding normal tissues as clearly observed by *in vivo* receptor imaging (Lamberts SWJ et al., 1996, *N. Engl. J. Med.*, **334**:246-254). Conceivably SSTRs in tumors behave differently due to a loss of normal receptor regulatory function, or to an alteration in the pattern and composition of the various subtypes expressed, or because of abnormal receptor signaling. The ability of SST to regulate SSTRs may provide a mechanism for targeting selective subtypes for diagnosis and therapy. For instance, upregulation of hSSTR1 and 2 by appropriate agonist treatment could be used for enhancing SSTR expression for receptor scans. Subtypes such as hSSTR3 and 5 which are extensively internalized could be targeted with selective α- or β-emitting SST radioligands for radiotherapy of certain SSTR positive human cancers.

While the invention has been described in connection with specific embodiments thereof, it will be understood that it is capable of further modifications and this application is intended to cover any variations, uses, or adaptations of the invention following, in general, the principles of the invention and including such departures from the present disclosure as come within known or customary practice within the art to which the invention pertains and as may be applied to the essential features hereinbefore set forth, and as follows in the scope of the appended claims.

I CLAIM:

1. A method for quantifying the amount of hSSTR1-5 receptors present on the cells, which comprises the steps of:

a) incubating membrane fractions from host cells expressing one of the five hSSTRs with saturating concentrations of a radioligand; and

b) determining the amount of hSSTR1-5 receptors present on the cell fractions of step a) in a saturation analysis.

2. A method for determining the ability of expressed hSSTR1-5 in host cells to be internalized which comprises the steps of:

a) culturing cells individually expressing hSSTR1-5 to about 90% confluency;

b) washing the cultured cells and incubating overnight at 4°C with a binding buffer containing [^{125}I] LTT SST-28;

c) washing the cells of step b) with binding buffer and warming to 37°C to initiate internalization;

d) removing surface-bound radioligand with acid wash; and

e) the internalized radioligand is measured as acid resistant counts in NaOH extracts of acid washed cells and the radioactive fractions are counted in a γ-spectrometer.

3. A method for determining the ability of expressed hSSTR1-5 in host cells to be upregulated in response to chronic agonist exposure, which comprises the steps of:

a) determining upregulation of hSSTRs of cells expressing hSSTR1-5 by culturing in a medium with SST agonist;

b) the cells are subjected to acid wash for to remove surface-bound SST; and

c) whole cell binding assay is carried out with a SST radioligand to determine total and nonspecific binding.

4. A recombinant host cell expressing the five hSSTRs receptors subtypes.

5. The use of the recombinant host cells of claim 4 for testing the ability of synthetic SST agonists or antagonists to induce receptor internalization or upregulation.

6. A method of targeted treatment of tumors based on the use of the internalization property of hSSTR3 and hSSTR5 subtypes, which comprises the use of α- or β -emitting SST radioligands for radiotherapy of tumors expressing a rich concentration of the hSSTR3 and hSSTR5 subtypes.

7. The method of claim 6, wherein the tumors are breast cancer tumors.

8. A method for increasing the sensitivity of receptors scans for the detection and diagnosis of tumors and inflammatory conditions in a patient based on the use of the upregulation property of hSSTR1, hSSTR2 and hSSTR4 subtypes, which comprises the steps of:

a) prolonged treatment of a patient with an agonist of hSSTR1 to increase the number of hSSTR1, hSSTR2 and hSSTR4 subtypes on the tumor and inflammatory conditions of said patient; and

b) visualization of said tumors and inflammatory conditions of step a) by administering a labeled hSSTR1 agonist.

9. The method of claim 8, wherein the agonist is SST-14.

10. A method for producing enhanced images in receptors scans by subtraction analysis for the detection and diagnosis of a tumor essentially expressing hSSTR1 in a patient based on the use of the upregulation property of hSSTR1 and the internalization property of hSSTR3, hSSTR4 and hSSTR5 subtypes, which comprises the steps of:

a) prolonged treatment of a patient with a hSSTR1 agonist to increase the number of hSSTR1 on the tumor of said patient;

b) administering agonist of hSSTR3, hSSTR4 and hSSTR5 subtypes for the internalization of hSSTR3, hSSTR4 and hSSTR5 subtypes expressed in tissues surrounding the tumor; and

b) visualization of said tumor by administering a labeled hSSTR1 agonist.

11. The method of claim 10, wherein the tumor is of pituitary origin.

12. A method for determining the potency of expressed hSSTR1-5 in host cells for binding to SST agonists and antagonists by competition analysis by displacement of membrane bound radioligand with known amounts of SST agonists.

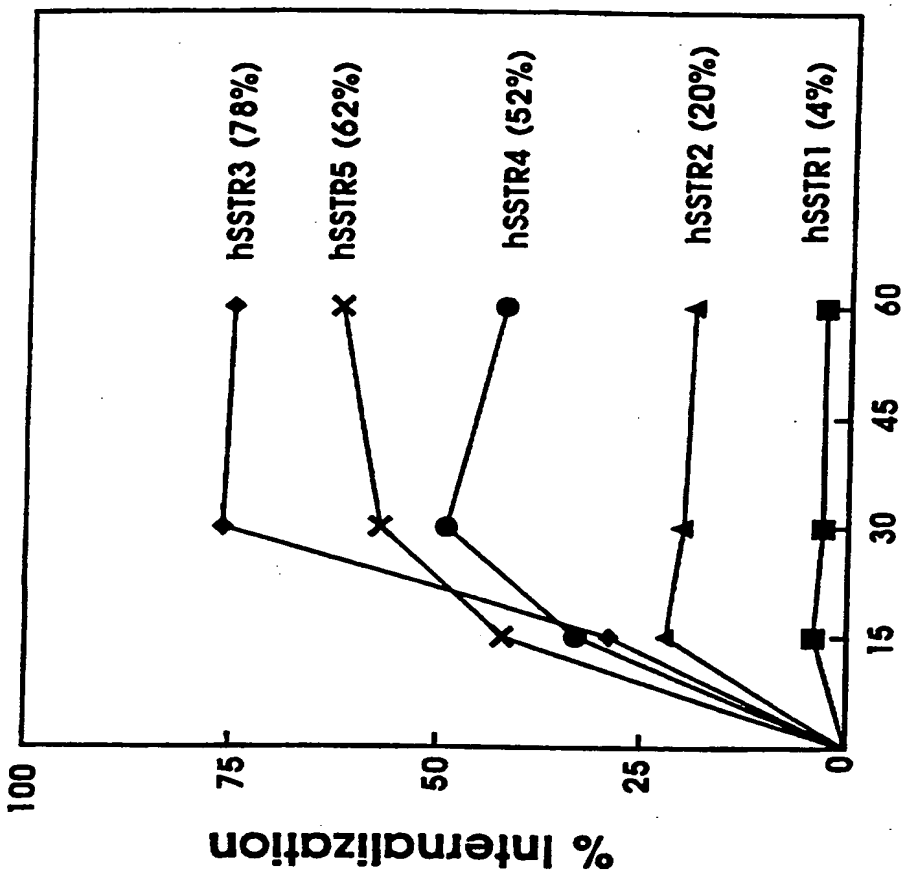
% Internalization
100
75
50
25
0
15
30
45
60
Time (min)
hSSTR3 (78%)
hSSTR5 (62%)
hSSTR4 (52%)
hSSTR2 (20%)
hSSTR1 (4%)

Fig. 1

2/4

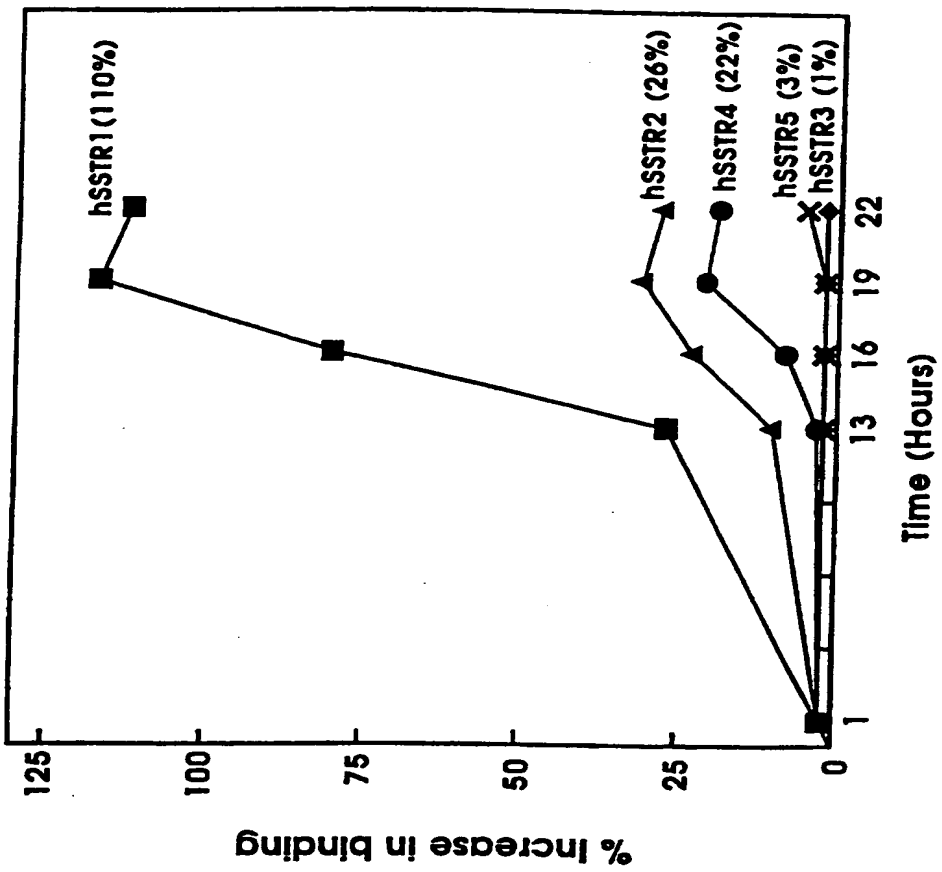
hSSTR1 (110%)
hSSTR2 (26%)
hSSTR4 (22%)
hSSTR5 (3%)
hSSTR3 (1%)
% Increase in binding
0
25
50
75
100
125
Time (Hours)
1
13
16
19
22

Fig. 2

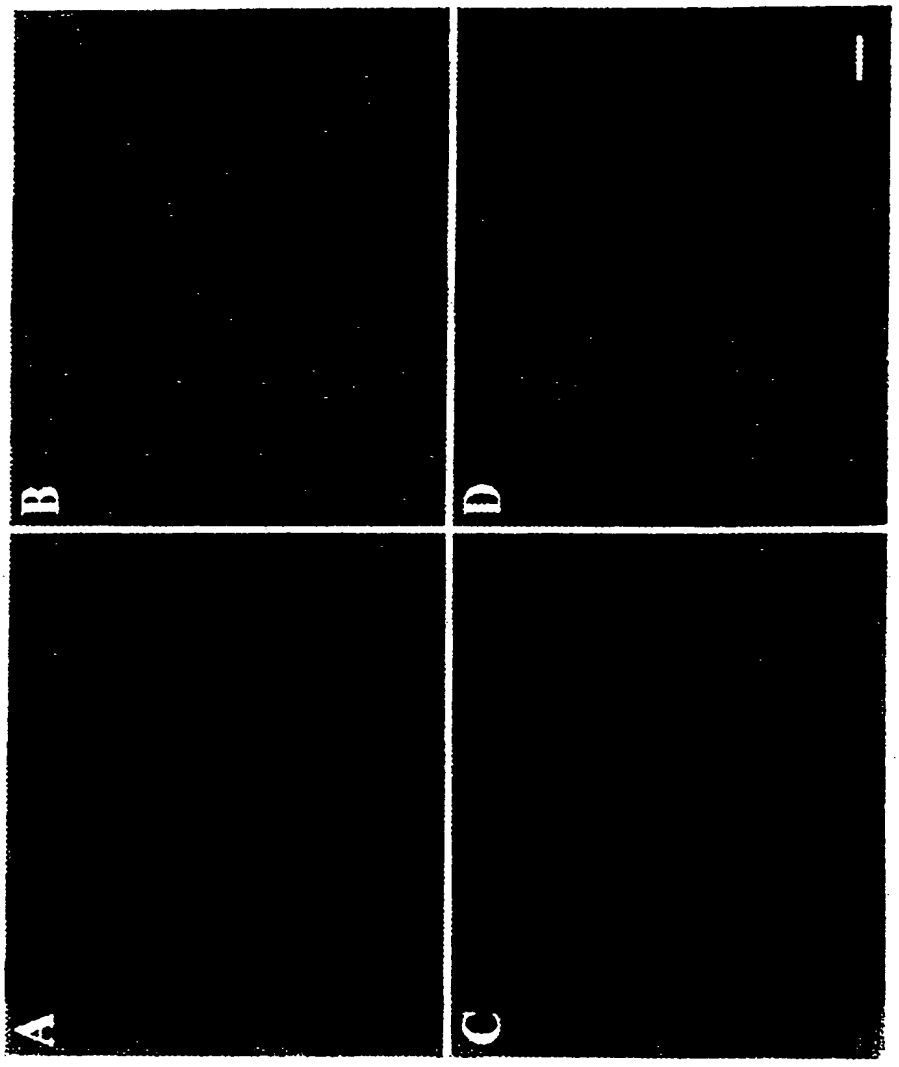

Fig. 3

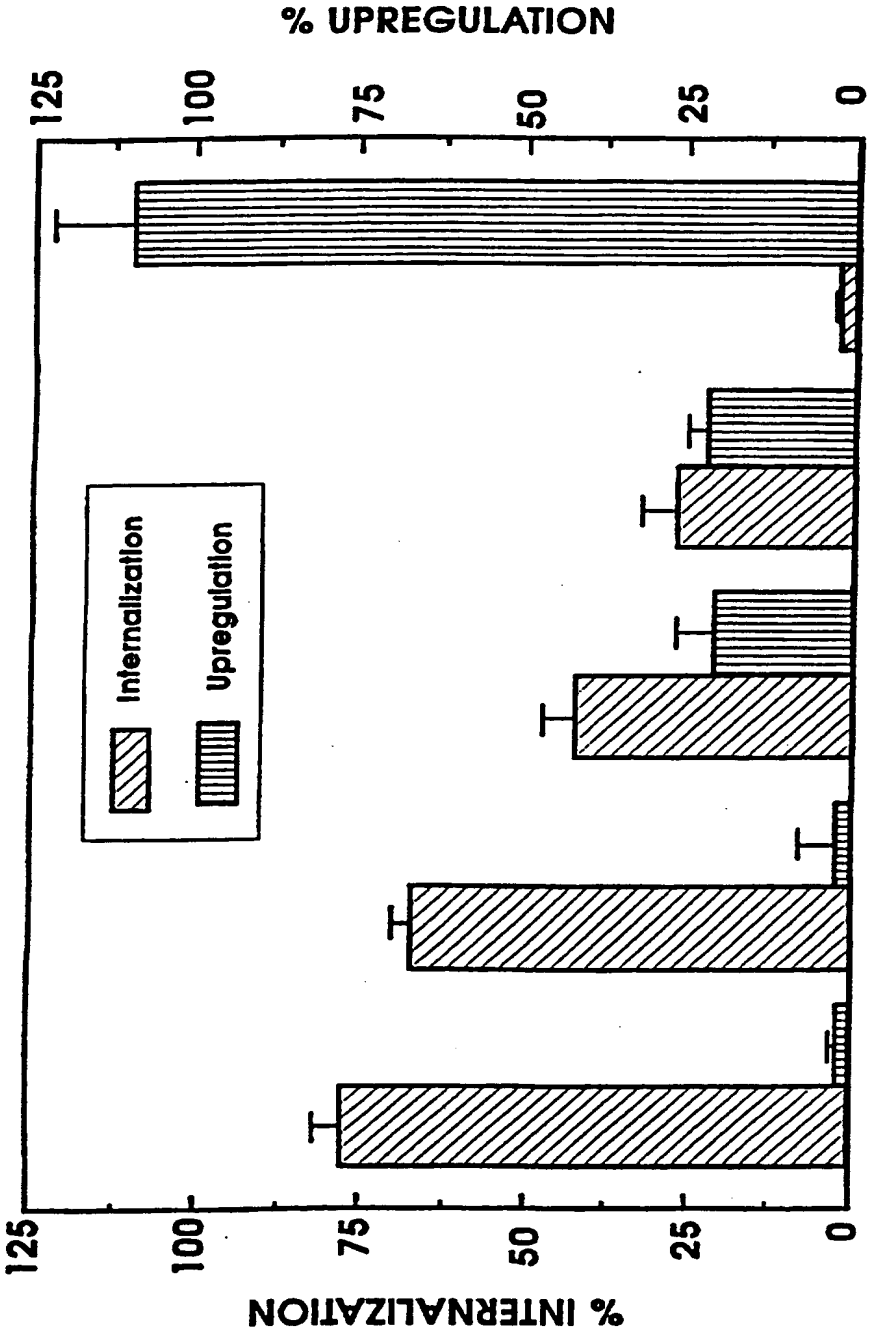
% UPREGULATION
125
100
75
50
25
0
Internalization
Upregulation
hSSTR1
hSSTR2
hSSTR4
hSSTR5
hSSTR3
% INTERNALIZATION

Fig. 4

INTERNATIONAL SEARCH REPORT

Interna al Application No
PCT/CA 97/00592

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 G01N33/74 G01N33/574 G01N33/50 C12N5/10 A61K51/08

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	PATEL ET AL.: "Expression of multiple somatostatin receptor genes in AtT-20 cells" THE JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 269, no. 2, 14 January 1994, pages 1506-1509, XP002049593 cited in the application see abstract see page 1507, right-hand column --- -/--	1-5,12

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
10 December 1997	23. 12. 97.

Name and mailing address of the ISA	Authorized officer
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INTERNATIONAL SEARCH REPORT

Interna..al Application No
PCT/CA 97/00592

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

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A	PATEL ET AL.: "All five cloned human somatostatin receptors (hSSTR1-5) are functionally coupled to adenyl cyclase" BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS, vol. 198, no. 2, 28 January 1994, pages 605-612, XP002049594 cited in the application see abstract ---	1-5,12
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